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The Prostate 30:160–166 (1997)

Muscarinic Cholinergic Receptors Promote Growth of Human Prostate Cancer Cells

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BACKGROUND. Recent evidence suggests that muscarinic receptors induce mitogenesis in cells capable of undergoing cell proliferation. Human prostate gland is innervated by the autonomic nervous system and muscarinic receptors have been localized in the prostate gland.
METHODS. Effects of carbachol (a stable analog of acetyl choline) on DNA synthesis of LNCaP cells (a human prostate cancer cell line) and primary prostate cells was examined. The DNA synthesis in the cultured cells was assessed using techniques of 3H-thymidine incorporation and bromodeoxyuridine (BrdU) incorporation immunocytochemistry.
RESULTS. Carbachol induced a significant increase in BrdU- and 3H-thymidine incorporation of LNCaP cells. The effect of carbachol was completely reversed by atropine, a selective muscarinic antagonist. Subtypes of muscarinic receptors mediating carbachol-induced DNA synthesis were identified using selective receptor subtype antagonists. Pirenzepamine and gallamine did not affect carbachol action on LNCaP cells but diphenylpyralamine, an M3 receptor antagonist, completely blocked carbachol-induced DNA synthesis. Carbachol also stimulated DNA synthesis in primary prostate cells. Prostate carcinoma (PC)-derived primary prostate cells displayed a dramatically greater response to carbachol (a ten-fold increase in DNA synthesis) as compared to benign prostate hypertrophy (BPH)-derived cells (a two-fold increase in DNA synthesis).
CONCLUSIONS. M3 receptors stimulate the proliferation of LNCaP cells, BPH-derived and PC-derived primary prostate cells. A dramatically higher response to carbachol by PC-derived prostate cells suggests that M3 receptors may be up-regulated in PC. M3 receptors may play a significant role in PC tumor growth and androgen-independent tumor progression. *Prostate 30:160–166, 1997.* © 1997 Wiley-Liss, Inc.

KEY WORDS: **muscarinic; growth; DNA synthesis; prostate cancer cells**

INTRODUCTION

The human prostate gland is innervated by the autonomous nervous system, and neurotransmitters released from this system may regulate vascular functions associated with secretion of seminal plasma constituents [1]. Receptors for some neurotransmitters such as adrenergic (alpha-1, alpha-2) and muscarinic acetylcholine receptors (mAChR) have also been localized in murine and human prostate glands [2–5] raising a possibility of their paracrine/autocrine action within the prostate gland. The biochemical as well as genetic studies suggest that mAChRs belong to a family of G protein-coupled receptors [6]. Structurally, these receptors are characterized by the presence of seven transmembrane domains, a ligand binding extracellular domain and a cytoplasmic portion [6,7].

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dogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 10 min. The amount of incorporated BrdU was determined using a monoclonal antibody directed against BrdU (Zymed Immunochemicals); the manufacturer's protocol was followed. The cells were stained with DAB (diaminobenzidine tetrahydrochloride) for color development, counterstained with hematoxylin, and dehydrated with a graded series of ethyl alcohol. Incorporated BrdU in the nuclei was identified as a dark brown stain.

BrdU Labeling Index

A total of 400 cells were counted on each slide. BrdU-positive cells were determined and the average BrdU labeling index was expressed as a percentage of the total number of cells scored.

Determination of Total 3H-Thymidine Incorporation Into DNA

Cultured LNCaP cells in log phase were seeded at 3×10^4 cells/well in 1 ml of RPMI-1640 growth medium (as described in cell culture above) in polylysine-coated 24-well culture plates. The growth rate of cells was slowed down by overnight incubation in low serum containing medium followed by 2-hr incubation in serum free basal medium (as described in BrdU incorporation). The cells were then treated with fresh basal medium containing various concentrations of agents, and the incubations were continued for 24 hr. Four hours prior to termination of the assay, the cells received 3H-thymidine (0.5 μCi/well). At the end of the incubation, the cells were washed twice with PBS containing 100 μM unlabeled thymidine, and solubilized in Triton X-100 (0.1% vol/vol in distilled water). The incorporated 3H-thymidine was quantified by liquid scintillation counting [15].

The results are expressed as cpm of 3H-thymidine incorporated per 3×10^4 cells ± SEM. The data were analyzed by t-test and the level of significance derived from Newman-Keul's test.

RESULTS

Effect of Carbachol on BrdU Incorporation in LNCaP Cells

LNCaP cells were labeled with BrdU, fixed and stained as indicated in the Materials and Methods section. The staining for BrdU in LNCaP cells was limited to the nucleus. Figure 1A presents a typical photomicrograph of BrdU-stained vehicle treated LNCaP cells. A total of 26 ± 2.69% stained for BrdU in the absence of mitogens. The cells treated with 100 μM carbachol showed a significantly higher percentage of BrdU staining (Fig. 1B). Approximately 48 ± 2.05% of carbachol-treated LNCaP cells displayed BrdU staining. This represented an 85% increase in BrdU labeling index in response to carbachol, and was statistically significantly different from vehicle-treated cells.

Effect of Carbachol on 3H-Thymidine Incorporation

Since the BrdU labeling index is only semiquantitative, additional characterization of the carbachol-induced DNA synthesis was done in 3H-thymidine incorporation assays. Effects of various concentrations of carbachol (0–1 mM) on 3H-thymidine incorporation in LNCaP cells were examined. The results presented in Figure 2 show that carbachol induced a dose-dependent increase in 3H-thymidine incorporation by LNCaP cells, and a maximal increase of 65% was observed at 100 μM (Fig. 2).

Effect of Carbachol on DNA Synthesis of LNCaP Cells Is Mediated Through mAChRs

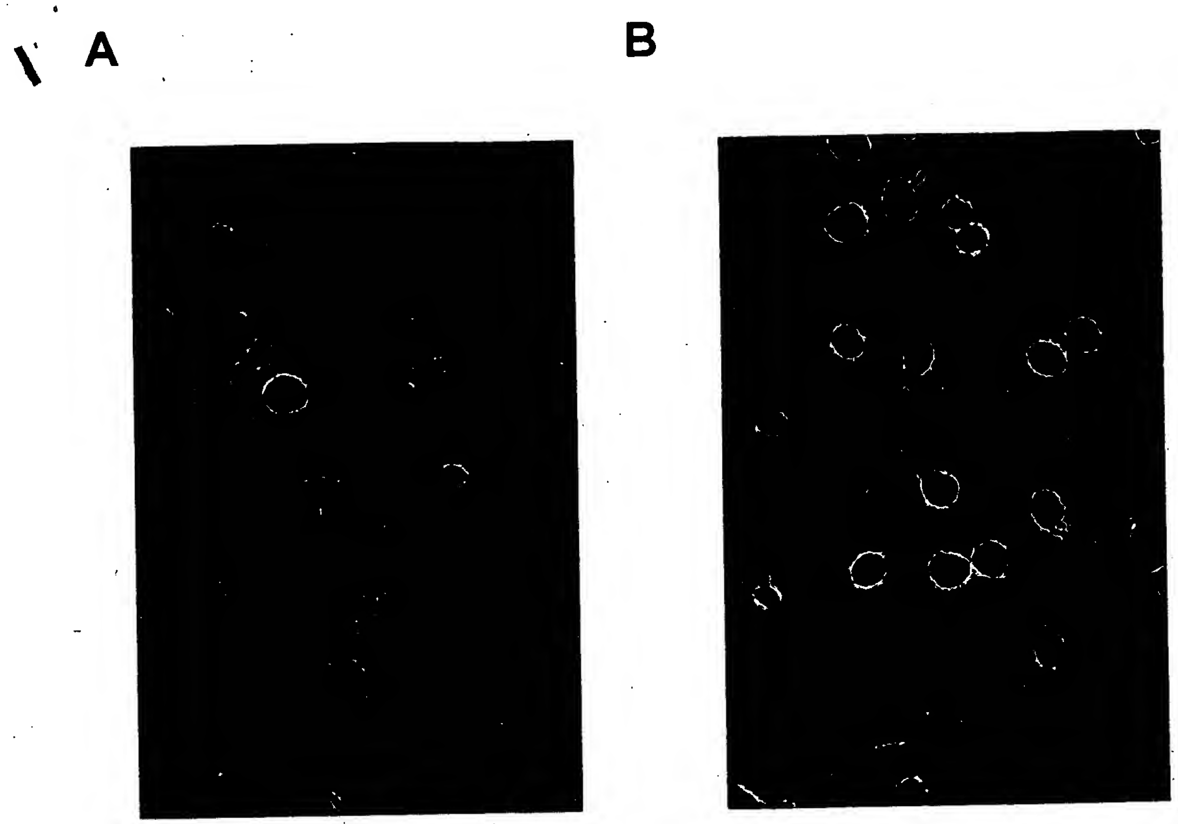
To demonstrate that the effect of carbachol was mediated by mAChRs, atropine, a specific muscarinic antagonist was used [16]. Atropine alone did not have any effect on 3H-thymidine incorporation, however, when co-administered with carbachol at equimolar concentrations (1 mM), it completely abolished the carbachol-induced increase in 3H-thymidine incorporation (Fig. 3).

Determination of mAChR Subtype in LNCaP Cells

To identify the mAChR subtype involved in carbachol-induced DNA synthesis, the effect of carbachol (1 mM) was examined in the presence of specific mAChR subtype antagonists. The results presented in Figure 4 suggest that pirenzapine (1 mM, an antagonist for m1 subtype [16,17]), had no effect on DNA synthesis, and did not affect the carbachol-induced DNA synthesis. Gallamine (1 mM, a specific m2 subtype antagonist [16,17]), also did not produce any effects either on DNA synthesis or carbachol response. However, diphenylpyraline (4-diphenylmethoxy-1-methyl-piperidine, 1 mM, a specific m3 antagonist [16,17]), did not affect DNA synthesis of LNCaP cells alone, but completely abolished carbachol-induced increase in DNA synthesis. These results suggest that carbachol induces DNA synthesis in LNCaP cells by activating m3 mAChR.

Effect of Carbachol on DNA Synthesis of Primary Prostate Cells

Since LNCaP is a cell line derived from metastasized prostate cancer, it is relatively homogeneous



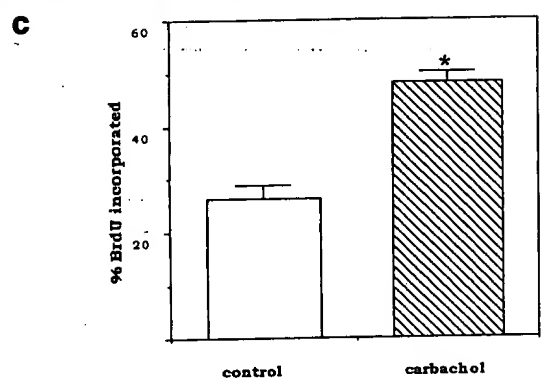


Fig. 1. Effect of carbachol on BrdU incorporation in LNCaP cells. **A:** A typical photomicrograph depicting BrdU incorporation in vehicle-treated LNCaP cells. LNCaP cells were cultured on poly-lysin-coated microscope slides and grown as described in the Materials and Methods section. The cells were incubated with 1 μg BrdU for 24 hr, fixed and immunolabeled as described in the Materials and Methods section. BrdU-labeled cells are characterized by dark nuclei (magnification: ×400). **B:** Effect of carbachol on BrdU incorporation. LNCaP cells were grown and labeled with BrdU as described in A, but in the presence of 100 μM carbachol. A significant increase in number of BrdU-labeled cells was observed. **C:** Figure 1C presents the pooled data from six independent experiments. Carbachol induced a significant increase in BrdU labeling index. $^*P < 0.05$ (t-test).

and may not contain other epithelial cell types of human prostate gland. To test whether carbachol exerts similar effects on primary prostate cells, we examined the effects of carbachol on primary cells derived from benign prostatic hypertrophy (BPH) and prostatic carcinoma (PC) explants. The results presented in Figure 5 show that carbachol induced a significant increase in DNA synthesis of primary prostate cancer cells from BPH as well as PC. While carbachol-induced increase in DNA synthesis was two-fold and comparable to the effect on LNCaP cells, carbachol-induced increase in DNA synthesis in PC-derived cells was ten-fold.

DISCUSSION

Previous findings by others have identified mAChR in stromal as well as epithelial compartments of the human prostate gland [2–5,10]. Present results have shown that carbachol induced a significant increase in BrdU incorporation and DNA synthesis by activating mAChRs. Since BrdU is incorporated into nuclear DNA during S-phase of the cell cycle [18], carbachol-induced increase in BrdU labeling index of LNCaP cells may be suggestive of proliferative response. The stimulatory effect of carbachol on DNA synthesis in LNCaP cells was further confirmed using a quantitative 3H-thymidine incorporation assay. The

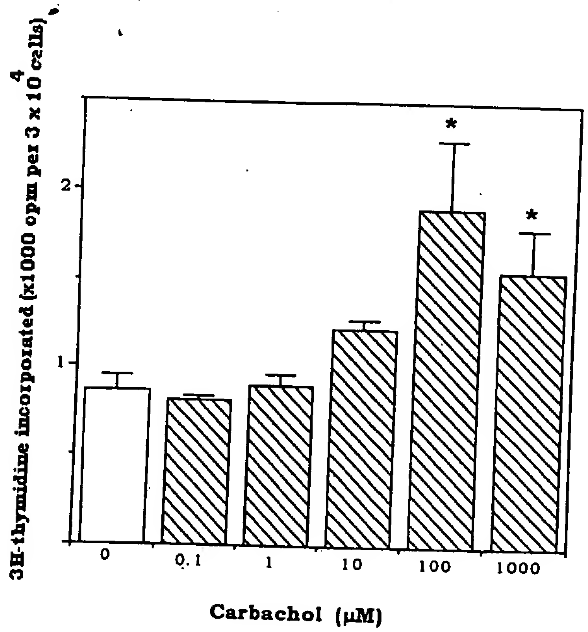


Fig. 2. Effect of carbachol on DNA synthesis in LNCaP cells: dose response. LNCaP cells were cultured in 24-well culture plates as described in the Materials and Methods section. The cells were tested with increasing concentrations of carbachol for 24 hr. The cells received 0.5 µCi 3H-thymidine for last 4 hr of incubation. Carbachol increased 3H-thymidine incorporation in LNCaP cells at concentrations of 10 nM and higher. *$P < 0.05$ (one way ANOVA and Newman-Keuls test).

carbachol-induced 3H-thymidine incorporation in LNCaP cells was dose-related, and was completely abolished by atropine, a specific antagonist of muscarinic cholinergics [16]. The results have further shown that carbachol-induced DNA synthesis was selectively blocked by diphenylpyraline [16,17], a specific m3 mAChR antagonist, and not by blockers of other receptor subtypes. These results suggest that mAChR receptor mediating mitogenic signal in LNCaP cells is of m3 subtypes. Since m3 mAChR is coupled to Ca^{2+}/phospholipid second messenger system [9], the signaling pathway may play an important role in transduction of mitogenic signals in prostate cancer cells.

Primary prostate cells from BPH as well as PC also responded to carbachol by showing increased DNA synthesis. However, there was a significant difference in magnitude of the response. Carbachol-induced increase in DNA synthesis of BPH-derived cells was comparable to LNCaP cells. In contrast, PC-derived prostate cells responded to carbachol with much greater, ten-fold, increase in DNA synthesis. These results raise a possibility that carbachol-responsive cell population is selectively amplified in PC-derived cells. Alternatively, elevated expression

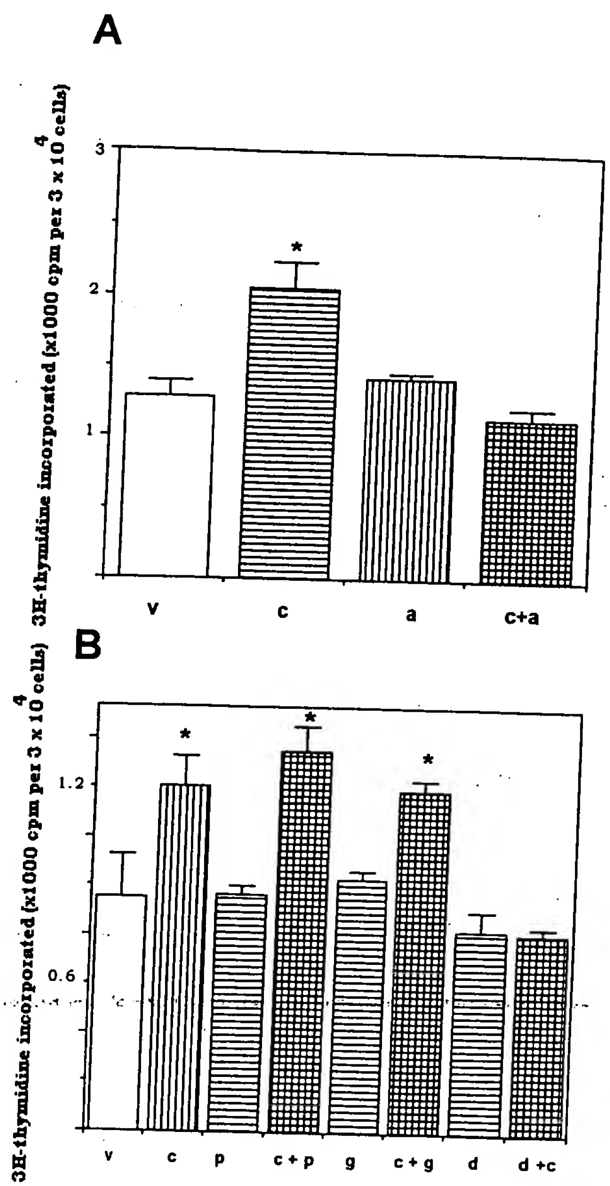


Fig. 3. Effect of muscarinic antagonists on carbachol-induced 3H-thymidine incorporation. A: Effect of carbachol on 3H-thymidine incorporation in LNCaP cells was examined in the absence and presence of atropine. The cells treated with 1 mM carbachol exhibited significantly higher 3H-thymidine incorporation (c) as compared to vehicle-treated cells (v). One millimolar atropine did not affect 3H-thymidine incorporation alone (a), but completely blocked carbachol-induced effect (c + a). *$P < 0.05$. B: Effect of selective antagonists of muscarinic receptor subtypes were tested on carbachol-induced 3H-thymidine incorporation. Pirenzepine and gallamine did not affect either baseline (p, g) or carbachol-induced 3H-thymidine incorporation (c+p, c+g) as compared to vehicle-treated cells (v). Diphenylpyraline also did not affect baseline DNA synthesis (d), but completely reversed the effect of carbachol (d+c). *$P < 0.05$.

of mAChR's receptors in PC-derived cell populations may have caused a magnified response. However, the receptor subtype(s) mediating the increased mi-

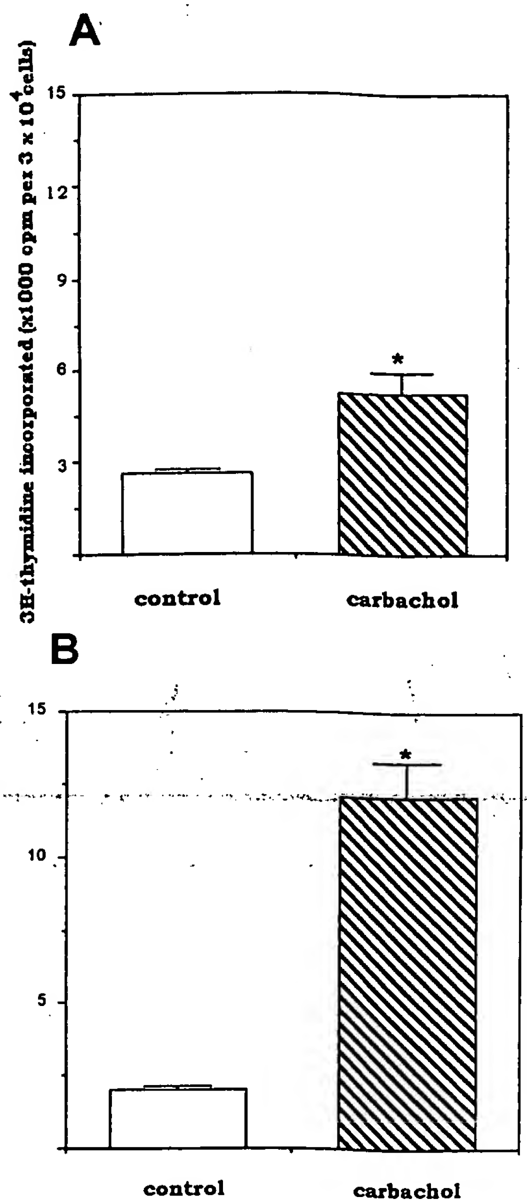


Fig. 4. Effect of carbachol on 3H-thymidine incorporation in primary prostate cells. **A:** Effect of 100 μM carbachol on 3H-thymidine incorporation in primary prostate cell derived from BPH tissue explants. A representative data from epithelial cell populations is presented as mean ± SEM for n=4. The carbachol-induced increase 3H-thymidine incorporation in cells derived from four BPH tissues ranged from 20–100%. **B:** Effect of 100 μM carbachol on 3H-thymidine incorporation in primary prostate cells derived from PC tissue explants. A representative data from epithelial cell populations is presented as mean ± SEM for n=4. The carbachol-induced increase 3H-thymidine incorporation in cells derived from three PC tissues ranged from 8- to 10-fold. *$P < 0.05$ (t-test).

togenic response in primary PC cells were not identified. Cell populations in basal lamina of glandular epithelium have been shown to bind tritiated muscarinic cholinergic receptor ligand and thus may form the target cell population for carbachol [4]. Since increased growth is also known to occur in this compartment of malignant prostate, the increased response of PC-derived prostate cells raises a strong possibility that mAChRs may be involved in the processes associated with development and/or progression of prostatic neoplasms [4,19]. A recent study has shown the m1 muscarinic receptor was predominant in epithelial compartment of PC, however m2, m3, and m4 receptors were also present in smaller concentrations [10]. Since m1 and m3 receptors activate Ca^{2+}/phospholipid signaling pathways, it is conceivable that they may mediate carbachol-induced proliferative response in primary PC cells, although m3 alone generated mitogenic response in LNCaP cells. Since LNCaP cells are not primary cells and may have undergone transformation during passaging, additional studies will be necessary to further characterize the receptor subtypes mediating mitogenic response in primary PC cells, determine the cell types expressing these receptors, and investigate whether this cell population is selectively amplified in cases of prostate adenocarcinoma.

Previous studies have identified muscarinic receptors in human prostate gland and have localized in stroma, as well as glandular epithelium [2–5]. Cholinergic nerve axons have also been localized in subepithelial region of human prostate [14]. Increase in sympathetic activity has been shown to activate muscarinic receptors and increase the flow of fluid from the prostate into the urethra in dogs [20]. The presence of muscarinic receptors has also been reported in cultures of smooth muscle cells from human prostate gland [3]. However, these receptors belong to the m2 subtype and are coupled to adenylate cyclase and are different from those that induce DNA synthesis in LNCaP prostate cancer cells [21]. These results suggest that isotypes of mAChRs in stromal and epithelial compartments of the prostate gland are different, are coupled to different second messenger systems and may have distinct functions in these regions. While mAChR receptors in the stromal compartment may mediate the actions associated with prostatic tension and exocrine functions, those in the epithelium may be associated with epithelial renewal under physiological circumstances and may promote growth in prostatic carcinoma.

In summary, our results demonstrate that carbachol increases DNA synthesis of human prostate cancer cells. This mitogenic response is mediated by m3 muscarinic receptors. A significantly higher response to carbachol by PC-derived cells suggests that expression of muscarinic receptors may be associated with increased growth of prostate cancer.

ACKNOWLEDGMENTS

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